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Fungal hyphae stimulate bacterial degradation of 2,6-dichlorobenzamide (BAM)

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ABSTRACT

Introduction of specific degrading microorganisms into polluted soil or aquifers is a promising remediation technology provided that the organisms survive and spread in the environment. We suggest that consortia, rather than single strains, may be better suited to overcome these challenges.

Here we introduced a fungal–bacterial consortium consisting of *Mortierella* sp. LEJ702 and the 2,6-dichlorobenzamide (BAM)-degrading *Aminobacter* sp. MSH1 into small sand columns. A more rapid mineralisation of BAM was obtained by the consortium compared to MSH1 alone especially at lower moisture contents. Results from quantitative real-time polymerase chain reaction (qPCR) demonstrated better spreading of *Aminobacter* when *Mortierella* was present suggesting that fungal hyphae may stimulate bacterial dispersal. Extraction and analysis of BAM indicated that translocation of the compound was also affected by the fungal hyphae in the sand. This suggests that fungal–bacterial consortia are promising for successful bioremediation of pesticide contamination.

1. Introduction

Pesticides used in agriculture or urban areas may be at risk of leaching to underlying groundwater resources. The most frequently detected pollutant in Danish groundwater is 2,6-dichlorobenzamide (BAM), a degradation product of the herbicide dichlobenil, the use of which has been banned in Denmark since 1997 (Thorling et al., 2010). Several water supply wells have been closed due to the detection of BAM at concentrations exceeding 0.1 μg l^{-1}, the threshold limit value for pesticides according to the regulation of the European Union on drinking water (EEC, 1980). Therefore, technologies for removing dichlobenil and BAM are needed to prevent them from leaching.

Bioremediation, especially bioaugmentation, has been suggested as a possible means of cleaning pesticide-polluted sites (Thompson et al., 2005). Various bacterial strains capable of degrading different pesticides have been identified, e.g. the BAM-degrading *Aminobacter* sp. strain MSH1 (Sørensen et al., 2007). There are, however, still major challenges that need to be overcome before bioaugmentation can be efficient (El Fantroussi and Agathos, 2005; Thompson et al., 2005). These challenges include: i) securing the survival of the introduced strains, ii) development of efficient tools for spreading degrader organisms in the polluted environment and iii) providing access for the microorganisms to the pollutants sorbed to the sediment and organic matter or trapped in micropores, e.g. small cracks or voids on the particle surfaces. It is difficult to ensure survival of the introduced strains (Van Veen et al., 1997; Pepper et al., 2002), since various factors like temperature, moisture content and nutrient availability in the new environment may differ from their original habitat (Vogel, 1996). Furthermore, studies have shown that bacteria applied to soil surfaces rarely are transported more than 5 cm into the soil without the help of a transport agent, such as percolating water (Edmonds, 1976; Madsen and Alexander, 1982). Even if the strains are successfully added to the environment, the pollutants are often only present at low concentrations and are not easily accessible for the degrader bacteria (Harms and Bosma, 1997; Wick et al., 2007). It has been suggested that fungal hyphae may function as a transport vector for bacteria capable of degrading hydrophobic PAHs (Furuno et al., 2010). Also, many bacteria can be found in the hyphosphere; thus they have better access to nutrients from fungal exudates, ensuring their survival in the environment (Boersma et al., 2010; de Boer et al., 2005). Therefore, adding fungal–bacterial consortia instead

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of single bacterial strains may be a way to overcome these challenges. Previous studies by Kohlmeier et al. (2005) and Wick et al. (2007) have moreover shown that fungal hyphae may facilitate transport of PAH-degrading bacteria in soil. The water film produced by some fungi around their hyphae allows a better transport of the bacteria present in the hyphosphere. Motile bacteria can utilise the water film as a transport vector enabling dispersal along the hyphae – a transport mechanism termed the fungal highway (Kohlmeier et al., 2005). Thus fungal growth may enable the bacteria to access areas and thereby potentially access pesticides they otherwise would not be able to reach. The water film may also facilitate translocation of the pollutant. The above mentioned studies have focused on hydrophobic compounds like PAHs and it has not been investigated whether fungus-mediated dispersal of bacteria is influencing degradation of hydrophilic compounds like many pesticides. The translocation of hydrophilic compounds along the fungal hyphae will also potentially differ from the transport of the hydrophobic PAHs.

In the present study we used the common soil fungus *Mortierella* and the BAM-degrading bacterium *Aminobacter* sp. MSH1. *Mortierella* is known to produce hydrophilic mycelia (Chau et al., 2010), a feature which has been shown to facilitate transport of motile bacteria (Kohlmeier et al., 2005). The aim of this study was to examine whether the presence of *Mortierella* sp. LEJ702 affected BAM degradation and mineralization by the motile *Aminobacter* sp. MSH1 in sand columns. Moreover, it was investigated whether BAM would be translocated along the hyphae of *Mortierella*. Our hypothesis was that presence of fungal hyphae facilitates translocation of both bacteria and BAM, thereby increasing the accessibility of BAM to the degrader bacteria, leading to an increased degradation.

2. Materials and methods

2.1. Chemical and media

[Ring-U-^{14}C]-2,6-dichlorobenzamide (BAM) (25.2 mCi mmol^{-1}) was purchased from Izotop (Institute of Isotopes Co., Ltd., Hungary). At the time of use, the standard had a radiochemical purity >99% as determined by Thin Layer Chromatography (TLC). A stock solution of radiolabeled BAM (3,000,000 DPM ml^{-1}) was prepared in acetonitrile.

Potato Dextrose Agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used for cultivation of *Mortierella* sp. LEJ702. Minimal medium (MSNC) containing 0.2 g L^{-1} Na-succinate (CAS 6106-21-4), 0.476 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} KNO_3 and 0.08% glycerol (Sørensen and Aamand, 2003) was used for growing *Aminobacter* sp. MSH1.

The liquid medium used for the experimental set up was buffered MilliQ water. The buffer contained 136 g L^{-1} KH_2PO_4 and 178 g L^{-1} $Na_2HPO_4 \cdot 2H_2O$. The buffer was mixed 1:99 with MilliQ water, giving a final phosphate concentration in the medium of 6 mM (pH 7).

Agar and media were sterilized by autoclaving at 121 °C for 30 min.

2.2. Strains/organisms

The BAM-degrading bacterial strain used in the experiments was the motile *Aminobacter* sp. MSH1 isolated and described by Sørensen et al. (2007). The strain was pre-grown from a −80 °C stock culture in MSNC medium at 20 °C on an orbital shaker (125 rpm). The cells were harvested in the exponential growth phase, determined by OD_{600nm} measurements. The cells were harvested by centrifugation at 8000 × *g* for 10 min and washed twice in buffered MilliQ water.

The fungus *Mortierella* sp. LEJ702 was isolated by Ellegaard-Jensen et al. (2013). It was grown and maintained at 8 °C on PDA plates.

2.3. Sand

The sand used was oven-dried quartz sand purchased from Saint Gobain Weber A/S (Optiroc, Randers, Denmark) with grain sizes between 0.3 and 1 mm. However, before experimental use it was sieved to remove particles between 0.3 and 0.6 mm. Thus, the sand used had a grain size between 0.6 and 1 mm giving a less densely packed matrix in the experimental set up. The sand was sterilized by autoclaving at 121 °C for 30 min. The water holding capacity (WHC) was determined (28.7%) according to Danish Standards; DS/ISO 14238-1.

2.4. Mineralization experiment

Mineralization experiments were set up in glass vials (24 mm Ø, 95 mm h) containing 500 µl PDA agar. Then either *Aminobacter* sp. MSH1, *Mortierella* sp. LEJ702, or the fungal–bacterial consortium was added to the agar. *Mortierella* was added as an agar plug (Ø = 4 mm) and the bacteria were added to reach 10^5 cells g^{-1} sand. Vials without microorganisms served as abiotic controls. The sand was weighed into portions of 10 g and added [ring-U-^{14}C]-BAM dissolved in acetonitrile, giving a concentration of 100 µg kg^{-1}. The sand was left for a few minutes in order for the acetonitrile to evaporate before adding buffered MilliQ water. The amount of water initially added in the treatments corresponded to 10%, 5%, 1.7% and 0% of WHC, respectively. A small glass tube containing 1 ml 1 M NaOH was added to each vial to trap evolved $^{14}CO_2$. A depiction of the set up can be seen in Fig. S1. The experiment was incubated at 20 °C. The NaOH was replaced approximately once a week and mixed (1:5) with Optiphase 'hisafe' 3 scintillation liquid (PerkinElmer Inc., Waltham, Massachusetts, USA). The amount of radioactivity in the alkaline solution was measured on a Wallac 1409 DSA Liquid scintillation Counter (PerkinElmer Inc., Waltham, Massachusetts, USA). All experiments were carried out in triplicate. The experiment was ended at day 63, when the mineralization had reached a plateau. At termination of the experiments, the columns were analysed for BAM residues using TLC (see below). To follow the removal of BAM and accumulation of degradation products a parallel series of vials was set up with the same inocula, but at a WHC of 1.7% only. Vials were harvested once a week for TLC analysis. The vials were harvested by removing the CO_2-trap and adding 6 ml MeOH-MilliQ water (50:50). The vials were then shaken vigorously for two hours, followed by centrifugation for 10 min at 200 × *g*. From the supernatant, 2 ml was transferred to Eppendorf tubes and centrifuged for 2 min at 13,000 × *g*. The supernatant was used for scintillation counting (500 µl) and for TLC analysis (40 µl).

2.5. DNA extraction and qPCR

To determine movement of *Aminobacter* DNA was extracted from the top sand (0.25 g), at termination of the mineralization experiment. DNA was extracted using the PowerLyzer PowerSoil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA) and immediately frozen at −20 °C. *Aminobacter* specific primers; Aminob151F (5′-ACCGTATACGTCCGATAGGA-3′) and Aminob585R (5′-CTCGGACTCTAGATTGCCAG-3′), targeting the 16S rDNA gene (Sjøholm et al., 2010) were used for quantitative real-time polymerase chain reaction (qPCR). Standards were prepared by adding a known number of *Aminobacter* MSH1 cells to the same sand as used for the experiments. Cell numbers were determined by OD measurement and direct cell counting. The DNA was extracted as described above and dilutions were made. The qPCR standard curve can be seen in Fig. S2. Each DNA extract was analysed in triplicates by qPCR. The amplification of DNA was done in a total reaction volume of 20 µl, consisting of 2 µl BSA, 10 µl SsoFast™ Eva Green Supermix (Bio-Rad, Hercules, California, USA) and 0.8 µl of each primer (10 pmol µl^{-1}). The conditions for the reactions were as follows: an initial denaturation step at 98 °C for 2 min, 40 cycles at 98 °C for 10 s, 60 °C for 30 s and 71 cycles at 60 °C for 10 s with a 0.5 °C increase per cycle. Amplification was performed on a Bio-Rad iCycler, MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) with a qPCR efficiency of 70.4%, r^2 = 0.992. The data from the qPCR were processed using the Bio-Rad iQ5 program (www.discover.bio-rad.com), and converted to cells per gram of sand.

2.6. Translocation of BAM

An experiment was set up to study whether *Mortierella* sp. LEJ702 facilitated the translocation of BAM through the sand. The experiment was set up in 20 ml sterile plastic syringes (Terumo Corporation, Belgium). The pistons were set at the 20 ml marking, 500 µl PDA and a *Mortierella* sp. LEJ702 plug (Ø = 4 mm) were added to the syringes. Five grams quartz sand was mixed with buffered MilliQ-water and [Ring-U-^{14}C]-BAM giving a concentration of 100 µg kg^{-1} as described above and added to the syringes followed by 5 g of unspiked quartz sand mixed with buffered MilliQ-water (see Fig. S3). The experiment was carried out at the same moisture conditions as in the mineralization experiment, adding buffered MilliQ-water initially corresponding to 10%, 5%, 1.7% and 0% of WHC. The syringes were sealed with rubber plugs and wrapped in parafilm and incubated at 20 °C for 63 days. Once a week the syringes were opened for aeration. At termination of the experiment the sand was pushed to the top of the syringe using the piston. The unspiked sand was divided into two equal size fractions, while the ^{14}C-spiked portion was kept as one portion. Radiolabeled carbon was extracted from the top fraction of the unspiked sand as described above with the modification that 3 ml MeOH:MilliQ-water was used for the extraction. After centrifugation, 500 µl of the supernatant was mixed with 3 ml Optiphase 'hisafe' 3 scintillation liquid (PerkinElmer Inc., Waltham, Massachusetts, USA) and measured on a Liquid Scintillation Analyzer, Tri-Carb 2810 TR (PerkinElmer Inc., Waltham, Massachusetts, USA).

2.7. Thin Layer Chromatography

A total of 40 µl of each sample was spotted onto a 10 × 20 cm silica gel 60 normal phase TLC plate (Merck, Darmstadt, Germany) and eluted with